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14. **ABSTRACT**
The contract supports studies to define the role of the PIM1 kinase in acquired resistance to chemotherapy by prostate cancer cells. Data to date for specific aim #1 define a signaling pathway induced by docetaxel, involving sequential steps of STAT3 activation, expression of PIM1, and activation of NFkB signaling. Blockade of this pathway by expression of dominant negative PIM1proteins blocks drug-induced upregulation of NFkB activity, and sensitizes cells to docetaxel. Other studies (specific aim #2) focus on identifying a mechanism through which PIM1 activates NFkB. We have unambiguously identified S937 as the major PIM1 phosphorylation site on the NFKB1/p105 precursor protein, through use of LCM/MS/MS analysis. Interestingly PIM2 is only a weak kinase for this site. Additional data (specific aim #3) have been generated to characterize a small molecule inhibitor of PIM1.

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Table of Contents

INTRODUCTION

Studies under this funded activity are focused on characterizing the role of the PIM1 gene in acquired resistance to chemotherapy drugs, by prostate cancer cells. The proposal included three specific aims: 1) to define a novel signal transduction pathway activated by docetaxel, 2) to characterize the mechanism through which PIM1 activates and regulates NFkB signaling, and 3) to explore genetic and pharmacologic means of inhibiting PIM1 activity or expression to enhance the sensitivity of prostate cancer cells to docetaxel and other chemotherapy drugs. Substantial progress has been made in each of these areas during the 01 year of support.

BODY

We will outline our progress through reference to the specific aims described above. The first specific aim was to outline a signal transduction pathway activated by docetaxel and involving upregulation of PIM1 expression. This pathway has been substantially defined. Using RWPE1 and RWPE2 (not shown) prostate cells, we noted that docetaxel treatment rapidly leads to an increase in expression of the PIM1 serine/threonine kinase. Expression becomes apparent at 3hrs after drug addition, peaks at 9-12hrs, and returns to baseline by 24hrs (Fig. 1). This increase in expression is accompanied by an increase in *pim-1* mRNA, as shown by real time-PCR analysis (Fig. 2). Thus the effects of docetaxel are primarily transcriptional or post-transcriptional.

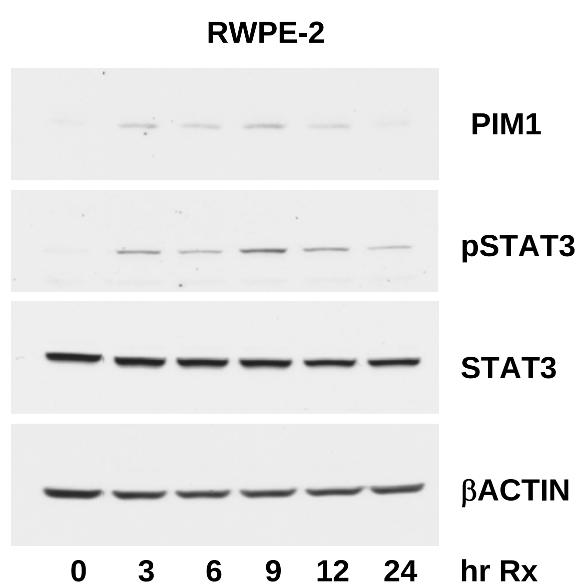
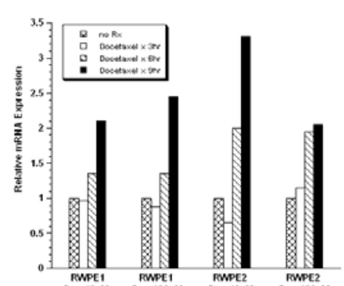


Fig. 1. Time course of PIM1, pSTAT3 expression

We next wanted to define mechanisms through which *pim-1* could be transcriptionally upregulated. Transcription of *pim-1* is known to be activated by STAT transcription factors and by NFkB transcription factors. We examined the time course of STAT3 activation after docetaxel treatment (Fig. 1), and noted that it paralleled the course of *pim-1* expression. We therefore suspected that docetaxel increased *pim-1* expression in a STAT3-dependent manner. This was directly demonstrated by use of decoy oligonucleotides (Fig. 3). Double-stranded DNA oligonucleotides matching a known STAT3 binding site blocked the drug-induced upregulation of *pim-1* expression, while a decoy based on a mutated (non-binding) STAT3 site did not. These data therefore establish a linear relationship among the following events: docetaxel treatment→ STAT3 activation→ *pim-1* expression.

Fig. 2. Expression of pim-1 mRNA after docetaxel treatment



We hypothesized that NFkB transcriptional activation would be a downstream event in this signal transduction pathway, because many chemotherapy drugs and other stressors are known to activate NFkB. We engineered RWPE2 cells to constitutively express a NFkB-dependent promoter/luciferase plasmid, and

found that docetaxel treatment increased NFkB transcriptional activity. We then transiently infected these cells with a *pim-1*-encoding retrovirus. *Pim-1* expression also consistently increased NFkB transcriptional activity (Fig. 4). To determine if the drug-induced increase in NFkB activity occurred in a *pim-1*-dependent manner, we then infected the reporter cell line with a retrovirus encoding a dominant-negative form of *pim-1*, pimNT81. The dominant negative *pim-1* cDNA completely blocked the drug-induced upregulation of NFkB activity, demonstrating that *pim-1* expression is a necessary upstream step in the drug-induced activation of NFkB (Fig. 5). In aggregate these studies establish a signal transduction pathway triggered by docetaxel treatment of RWPE2 prostate cancer cells.

Fig. 3. STAT3 decoy oligonucleotide blocks pim1 increase after docetaxel treatment

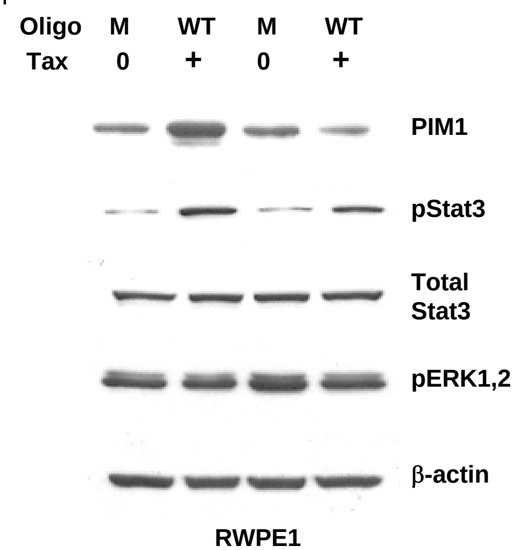


Fig. 4. Pim1 transduction increases NFkB transcriptional activity

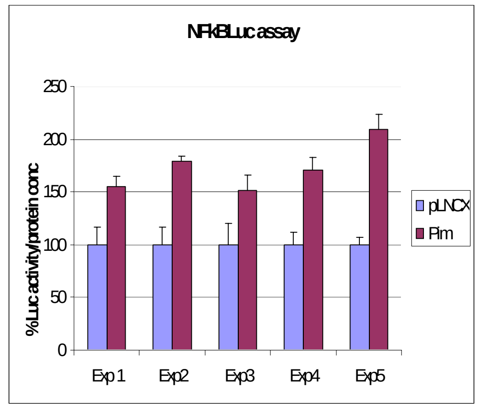
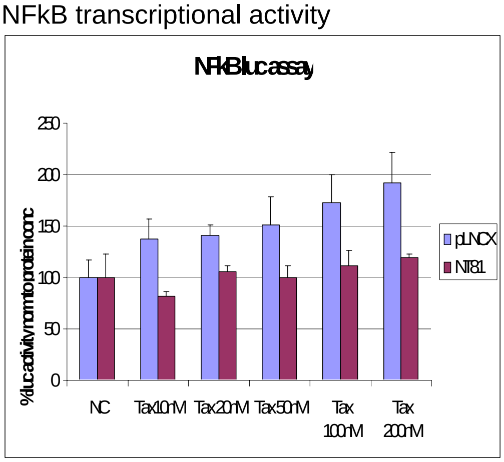
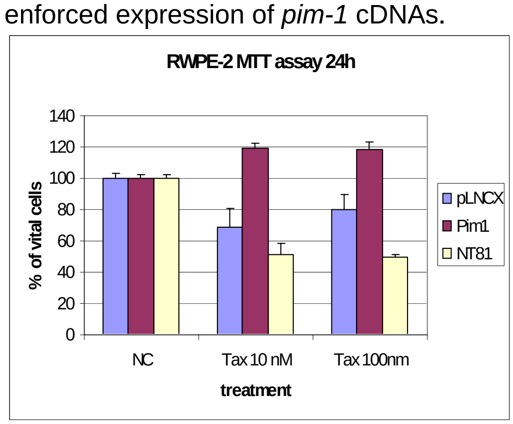


Fig. 5. Dominant negative PIM1 (NT81) blocks docetaxel-induced activation of NFkB transcriptional activity



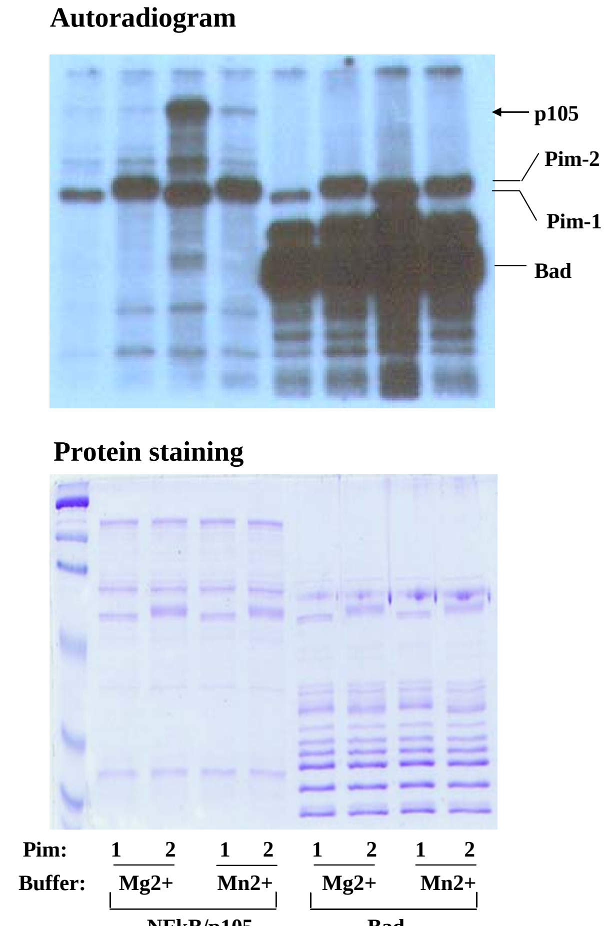
To determine if this pathway modified drug toxicity, we examined the effects of enforced expression of wild-type or NT81 *pim-1* cDNAs of docetaxel cell kill (Fig. 6). Docetaxel produced dose-dependent cell kill in RWPE1, 2 cells. Enforced expression of wild-type *pim-1* cDNA markedly reduced cell death. In contrast, expression of the dominant negative NT81 cDNA enhanced cell death after docetaxel treatment. These data demonstrate that *pim-1* expression can modulate drug-induced cell death, and demonstrate that the survival pathway described above is a legitimate target for pharmacologic intervention. These data will be presented at the 2006 AACR meeting in poster form (1).

Fig. 6. Modulation of docetaxel cell kill by enforced expression of *pim-1* cDNAs.



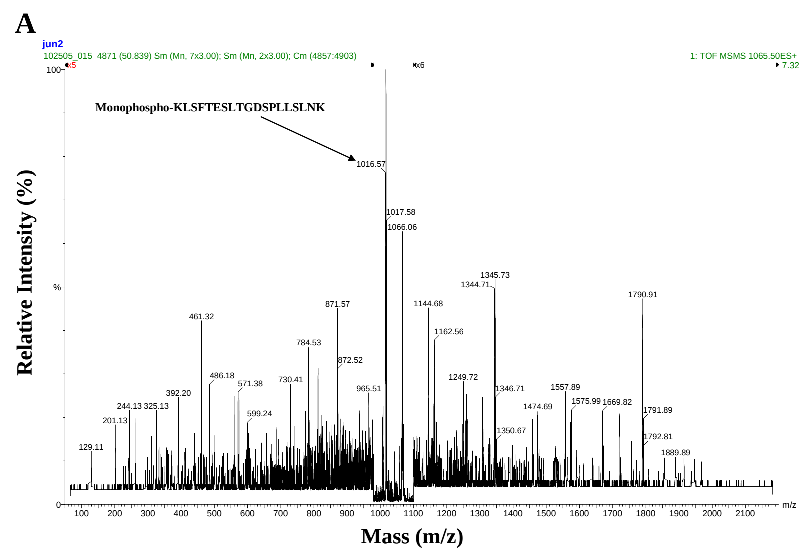
The goal of specific aim #2 was to define pathways through which the PIM1 kinase could activate NFkB transcriptional activity. We had hypothesized that PIM1 would phosphorylate the NFKB1/p105 precursor protein on serine-937, leading to proteolytic cleavage of the protein with release of active p50 protein as well as other sequestered NFkB components and the TPL2 kinase. A major goal of this specific aim was to identify the phosphorylation site on p105. We have used a variety of biochemical methods to accomplish the unambiguous identification. We initially expressed the full-length p105 protein in bacteria. This was reacted in a variety of *in vitro* kinase reactions with recombinant PIM1 or PIM2 enzymes. PIM1 strongly phosphorylated p105, but only in the presence of manganese, not magnesium. PIM2 was a much weaker kinase (Fig. 7).

Fig. 7. PIM1 phosphorylates p105



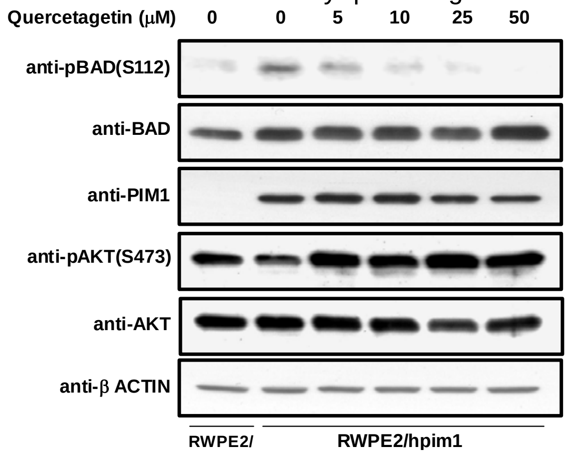
To demonstrate the site of phosphorylation we used mass spectroscopy of tyrpsin-digested fragments of p105 that had been phosphorylated in vitro. We had previously demonstrated that PIM1-dependent phosphorylation happens exclusively on serine. Fragments were separated by LC/MS/MS analysis and mass/charge rations were determined. The predicted peptide fragment that would result from phosphorylation at serine-937 was recovered, with a mass of 1016 (Fig. 8). Since there are several potential phosphorylation sites within this peptide, we proceeded to sequence the peptide with mass spectroscopy. Only the fragment corresponding to phosphoserine-937 was not recovered. These data unambiguously demonstrate that the major phosphorylation site of PIM1 on p105 is serine-937. We also found evidence by MALDI-MS that serine-851 may also be phosphorylated by PIM1. These data have not yet been confirmed by LC/MS/MS analysis.

Fig. 8. Mass spectroscopy of p105 phosphorylated by PIM1



The third specific aim proposed to use small molecule inhibitors of the PIM1 kinase as molecular probes to determine their effect on docetaxel sensitivity. We have submitted a report describing one such molecule, the flavonol quercetagetin (2). We have demonstrated that quercetagetin in a moderately potent (IC_{50} = 340nM, specific, and cell-permeable inhibitor of PIM1 activity in prostate cancer cells. Key data include the demonstration that quercetagetin in competitive with ATP. A crystal structure of PIM1 in complex with quercetagetin, or with three other flavonoids, has been determined. We have also shown that quercetagetin is able to inhibit the activity of the PIM1 kinase in prostate cancer cells at an IC_{50} of about 5.5μM. Interestingly the activity of the AKT kinase is not inhibited at all under these conditions (Fig. 9). RWPE2 prostate cancer cells treated with quercetagetin develop morphologic changes consistent with differentiation or senescence, accompanied by profound growth inhibition, at concentrations that inhibit PIM1 kinase activity (Fig. 10).

Fig. 9. PIM1 kinase activity in RWPE2/hpim1 cells is blocked by quercetagetin



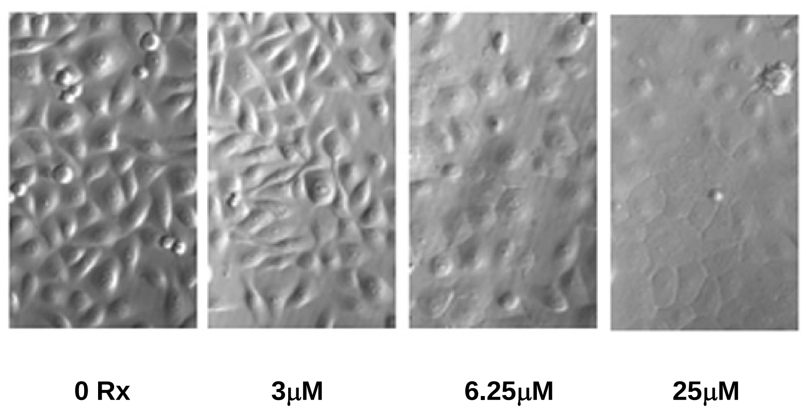


Fig. 10. Differentiation of RWPE2 cells in response to quercetagetin

KEY RESEARCH ACCOMPLISHMENTS

- Definition of a novel survival pathway activated by docetaxel treatment, and involving sequential activation or expression of STAT3, PIM1, and NFkB components.
- Identification of serine-937 as the major phosphorylation site for PIM1 on the p105/NFKB1 precursor protein
- Identication of quercetagetin as a moderately potent and specific, cell-permeable PIM1 kinase inhibitor
- Abstract accepted for presentation at the annual AACR meeting, Washington DC, April, 2006

REPORTABLE OUTCOMES

None in 01 year

CONCLUSIONS

Our data demonstrate that PIM1 is a critical component of a survival/stress pathway activated by docetaxel treatment of prostate cancer cells. This pathway leads to activation of NFkB-dependent transcription, possibly by phosphorylation of p105/NFKB1 by PIM1 at serine-937. Targeting PIM1 kinase activity with quercetagetin, or other PIM1 kinase inhibitors, may lead to additive or synergistic cell kill following docetaxel treatment.

REFERENCES

1. Zemskova M, Sahakian E, Lilly M: The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death (abstract #2777), approved for presentation at 97th Annual Meeting of AACR, Washington, DC, April 2006.

2. Holder S, Zemskova M, Bremer R, Neidigh JW, Lilly MB: Characterization of a potent and selective small-molecule inhibitor of the PIM1 kinase (submitted to Molecular Cancer Therapeutics, 2006).

APPENDIX

Research data are presented throughout the body of this report.
The appendix contains two items:

1. AACR abstract #2777, approved for presentation at the 97th Annual Meeting, April, 2006, entitled "The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death" by M. Zemskova, E. Sahakian, M. Lilly.
2. Curriculum vitae for Michael Lilly, MD

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The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death.

Short Title:
PIM1 and docetaxel-induced death

Author Block: *Marina Zemskova, Eva Sahakian, Michael B. Lilly.* Loma Linda University, Loma Linda, CA

The PIM1 serine-threonine kinase is a true oncogene that mediates survival and proliferation signals in human neoplasms, including leukemias and prostate adenocarcinomas. Enforced expression of PIM1 has been shown to enhance resistance of cells to cytotoxic agents and ionizing radiation. To examine potential pathways through which PIM1 produces drug resistance, we examined RWPE1 and RWPE2 prostate epithelial cells treated with docetaxel, a taxane used for treatment of prostate cancer. Cells treated with docetaxel demonstrated an increase in PIM1 protein and pim-1 mRNA 3-12 hrs after drug exposure. We then sought to establish upstream and downstream effectors of PIM1 expression. Docetaxel also induced expression of phosphoSTAT3 with similar kinetics. Drug-induced upregulation of PIM1 expression was abolished when cells were transfected with STAT3 decoy oligonucleotides, demonstrating that STAT3 activation by docetaxel is required for drug-induced upregulation of PIM1. Docetaxel treatment, and infection with a PIM1 expressing retrovirus, both induced activation of NFkB transcriptional activity in RWPE2 stably transfected with an NFkB/luciferase reporter plasmid. However, when a dominant negative PIM1 protein (NT81) was introduced by retroviral transduction, drug induced activation of NFkB activity was abolished. Retroviral transduction of wild-type PIM1 or dominant-negative (NT81) PIM1 increased or decreased survival of RWPE1 and RWPE2 cells treated with docetaxel. These data establish a survival pathway (drug → STAT3 → PIM1 → NFkB) induced by docetaxel and capable of impairing drug cytotoxicity. Targeting the PIM1 kinase, along with STAT3 and NFkB, may be a viable approach to enhancing cell kill by cytotoxic drugs such as docetaxel.

Author Disclosure Block: M. Zemskova, None; **E. Sahakian**, None; **M.B. Lilly**, None.

Additional Disclosures (Complete):

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Date [mm/dd/yyyy]: : 11/10/2005

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Organizations:	Fellow, American College of Physicians Member, American Society of Hematology Member, American Society for Bone Marrow Transplantation Member, American Society for Gene Therapy
National Professional Responsibilities	Member, ad hoc study sections for NIH: 1987 Diagnostic Radiology 1988 Experimental Therapeutics Member, site visit team for program project

Dr. George Hahn, PI; Stanford University
1988, 1989
Member, site visit team for program project
Dr. Bayard Clarkson, PI, Memorial-Sloan
Kettering Inst., 1997

Special Local Responsibilities

Member, Scientific Review Subcommittee
SVAMC, 1993, 1994, 1997
Member, Research & Development Committee
SVAMC, 1994, 1995
Member, Hospice Advisory Committee
SVAMC, 1994, 1995
Board Development Committee, Leukemia & Lymphoma Society (Southern California Chapter), 2003

Consultant

Cetus Corporation (1986)
EncorePharma (2001-present)
Myriad Genetics (2002-present)
Exelixis Pharmaceuticals (2005-present)

GRANTS & CONTRACTS (PRINCIPAL INVESTIGATOR) *Note: This listing does not include multicenter clinical trials in which Dr. Lilly was the local principal investigator.*

National Institutes of Health F32CA27980 *Hyperthermia of animal and human tumors*; 7/80-6/82

National Institutes of Health R01CA18138-*11* *Prediction of thermal tolerance by in vivo NMR spectroscopy*; 7/82-6/83

National Institutes of Health R01CA36790 *Assessment of hyperthermia by in vivo* ^{31}*P-NMR spectroscopy*; 9/84-9/87

Cetus Corporation *Characterization of a human granulocyte CSF*; 7/85-6/86

National Institutes of Health R01CA45672 *Cytokine signaling in myeloid leukemia*; 9/87-10/98

VA Merit Review Award *Non-protein hematopoietic agents*; 10/90-4/97

March of Dimes Birth Defects Foundation *Characterization of a 28kd protein related to G-CSF*; 7/93-6/96

Lymphoma Research Foundation of America *Mechanism of action of the pim-1 oncogene*; 7/95-7/96

Roche Pharmaceuticals *Preclinical study of Roferon and bryostatin 1 in a melanoma model*; 1/98-12/99

Department of Defense, National Medical Technology Testbed #76-FY99: *Cell-permeable proteins for cell regulation.* 12/99 – 7/02

Leukemia Society of American Translational Award *Propionic Acid Analogues for CLL*. 9/1/01 – 8/31/05

Celgene Corporation, *Phase I-II trial of combined GM-CSF (sargramostim) and thalidomide for hormone-refractory prostate cancer* (5/02-5/04).

National Institutes of Health R03CA107820 *Molecular Targets of NSAIDs in Prostate* Cancer; (5/1/04 – 4/30/07)

Department of Defense, CDMRP Prostate Cancer Program PC040635 *Pim-1: A Molecular Target to Modulate Cellular Resistance to Therapy in Prostate Cancer* (10/04 – 10/07)

Pharmion Corporation, *Use of azacytidine to reverse silencing of GST-p1 in early prostate cancer.* (10/05 – 10/07)

GRANTS and CONTRACTS (Co-investigator)

National Institutes of Health R01CA097043 *Molecular pathology of 2-deoxy-5-azacytidine*; L. Sowers, PI; Michael Lilly, co-investigator (10% FTE). 7/1/03 – 6/30/08

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